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# Early detection of burn induced heterotopic ossification using transcutaneous Raman spectroscopy☆,☆☆

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ABSTRACT

*Introduction:* Heterotopic ossification (HO), or the abnormal formation of bone in soft tissue, occurs in over 60% of major burn injuries and blast traumas. A significant need exists to improve the current diagnostic modalities for HO which are inadequate to diagnose and intervene on HO at early time-points. Raman spectroscopy has been used in previous studies to report on changes in bone composition during bone development but has not yet been applied to burn induced HO. In this study, we validate transcutaneous, in-vivo Raman spectroscopy as a methodology for early diagnosis of HO in mice following a burn injury.

*Methods:* An Achilles tenotomy model was used to study HO formation. Following tenotomy, mice were divided into burn and sham groups with exposure of 30% surface area on the dorsum to 60° water or 30° water for 18 s respectively. In-vivo, transcutaneous Raman spectroscopy was performed at early time points (5 days, 2 and 3 weeks) and a late time point (3 months) on both the tenotomized and non-injured leg. These same samples were then dissected down to the bone and ex-vivo Raman measurements were performed on the excised tissue. Bone formation was verified with Micro CT and histology at corresponding time-points.

*Results:* Our Raman probe allowed non-invasive, transcutaneous evaluation of heterotopic bone formation. Raman data showed significantly increased bone mineral signaling in the tenotomy compared to control leg at 5 days post injury, with the difference increasing over time whereas Micro CT did not demonstrate heterotopic bone until three weeks. Ex-vivo Raman measurements showed significant differences in the amount of HO in the burn compared to sham groups and also showed differences in the spectra of new, ectopic bone compared to pre-existing cortical bone.

*Conclusions:* Burn injury increases the likelihood of developing HO when combined with traumatic injury. In our in-vivo mouse model, Raman spectroscopy allowed for detection of HO formation as early as 5 days post injury. Changes in bone mineral and matrix composition of the new bone were also evidenced in the Raman spectra which could facilitate early identification of HO and allow more timely therapy decisions for HO patients.



## Introduction

Heterotopic ossification (HO), the abnormal development of bone in soft tissues, is a clinically devastating sequela of trauma, burn, and orthopedic surgery which lacks a reliable method for early diagnosis. More than 60% of major burn patients, 65% of major combat injury patients, and 10% of patients who have invasive surgery develop HO [1,2]. Without early detection or intervention, progression of HO can lead to severe long-term effects, including restricted joint mobility, severe pain, and nerve entrapment. Current techniques for diagnosis rely on clinical examination and radiographic imaging modalities that have low sensitivity to the incremental progression of mineralization associated with the early stages of HO [3–5]. Therefore, an urgent need exists to develop novel screening techniques to visualize and detect the onset and progression of HO with high sensitivity and specificity. The exact mechanism of HO is still largely unknown, though it is thought to require osteogenic precursor cells, an inciting incident, and a permissive niche [2,6].

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The formation of HO begins within days to weeks of the inciting event, however the exact timing of its earliest occurrence is unknown. Clinically, symptoms are non-specific and consist of swelling, edema, and pain. Patients may have an increase in alkaline phosphatase (AP) levels, however AP levels are also elevated in global trauma and liver disease. Currently, if HO is suspected clinically, radiography is obtained (X-ray or CT). Once visible through these radiographic modalities, however, the disease has already spread beyond the point where it can be treated with oral medications such as non-steroidal anti-inflammatory agents or bisphosphonates such as etindronate [7]. Presence of HO requires invasive surgical resection which has significant risk and leaves over 75% of patients with functional deficits [1,8–10]. Thus, patients would greatly benefit if physicians had access to a point of care, non-invasive, sensitive imaging technique that could be done routinely to detect HO.

The vast number of imaging modalities used to diagnose HO and the lack of consensus guidelines for HO diagnosis is indicative of the current shortcomings of available imaging technologies. Currently three-phase bone scintigraphy is the most sensitive imaging modality of early HO detection [1]. Despite its sensitivity to detect HO, this imaging modality has a high level of variability and false positives which are especially prominent after trauma or fractures where there is a significant amount of inflammation or callus at the injury site [4,11]. Similarly, ultrasound technology has been shown to detect HO sooner than conventional radiography and can be used in a point of care manner [3,12]. Like 3-phase scintigraphy, ultrasound cannot distinguish new bone formation from a normally healing fracture callus. Radiographic techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) provide highly detailed anatomic representation of late stage HO, however, these modalities cannot detect early stages of the disease process. Thus, despite being helpful for surgical planning of HO extirpation, current radiographic techniques do not allow for early diagnosis of the disease and the potential institution of therapeutic remedies that can impede or even prevent the process from becoming clinically significant. In summary, current imaging modalities, though helpful in late diagnosis, are inadequate to help clinicians detect early HO development or aid in its interventional treatment.

We hypothesize that Raman Spectroscopy offers a novel diagnostic technology that is non-invasive, informs surgeons about the extent of the disease process for surgical planning, and also provides the opportunity for early diagnosis which is currently unavailable [13,14]. These unique features may enable early clinical intervention and facilitate focused treatment paradigms designed to halt the progression of HO and its associated morbidities. A single report of the application of Raman spectroscopy to characterize HO [2] describes mineralized collagen found in debrided combat wound tissue at a time before it was detectable visually or by other standard modalities. However, this imaging was performed on tissue samples following excision rather than in a non-invasive manner.

Development of non-invasive imaging techniques to detect early signs of HO would allow for improved prophylactic and treatment strategies. The aim of this study was to establish quantifiable metrics of non-invasive, transcutaneous Raman spectroscopy in the early formation and progression of HO in a mouse model of burn induced HO. We hypothesized that Raman would have the ability to distinguish heterotopic bone from native bone, that the detected signal from the heterotopic bone would intensify with maturity over time, and that these metrics could be validated with Micro CT, ex-vivo Raman, and histology.

## Methods

### *Burn injury and Achilles tenotomy models*

The procedure was performed according to a previously established method to produce partial-thickness burn injury [15–17]. In brief, 9 male, C57BL/6 mice 7–8 weeks old were anesthetized with a 50 mg/kg intraperitoneal (ip) injection of sodium pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL). Dorsal hair was closely clipped. Each mouse was placed in an insulated, custom-made mold, which exposed the dorsal region over 30% of the total body surface area. Partial-thickness scald burn injury was achieved by placing the exposed skin of the mouse in a 60 °C water bath for 18 s ($n=3$). Sham burn animals received the same treatment except they were immersed in room temperature water (30 °C, $n=6$).

The burn wound was scrub debrided with dry sterile gauze and each animal was resuscitated with 1 mL Ringer's lactate solution IP injection and 0.5 mL subcutaneous injection. After drying, an occlusive dressing of sterile Tegaderm HP (3M HealthCare, St. Paul, MN) was applied to prevent wound contamination. All mice received an Achilles tenotomy on the left leg after burn or sham injury. A 1 cm incision was made on the lateral aspect of the Achilles tendon with a surgical knife. Subsequently, the Achilles tendon was exposed from its origin on the distal end of the gastrocnemius to the insertion at the calcaneus. The Achilles tendon was then divided sharply at its midpoint. The incision was then closed with absorbable sutures. Experiments were performed in accordance with National Institute of Health guidelines and prior approval was obtained from the University of Michigan Animal Care and Use Committee (IACUC #0001553).

### *Raman spectroscopy*

The fiber optic probe system used for these experiments has been previously described [14]. Spectroscopic measurements were completed at four time points (5 days, 2 weeks, 3 weeks, and 3 months) longitudinally ($n=3$ burn, $n=6$ sham). Briefly, the anesthetized mouse is placed on the probe with its left hind leg positioned in the probe assembly. The optical fibers are positioned so that they just contact the animal's skin. Prior to positioning the mouse for Raman measurement, hair was removed from the leg using a depilatory cream (Nair), and glycerol was topically applied as optical clearing agent [18].

Raman measurements were made with a portable system (Rxn-1, Kaiser Optical Systems, Inc., Ann Arbor. MI). A calibration tool (HCA, Kaiser Optical Systems) was used daily for white light correction and calibration of detector wavelength axis. The HCA contains a white light source for calibration of the detector wavelength response and a neon discharge lamp for calibration of the spectrograph wavelength scale. A fluorocarbon excitation fiber was used to correct for skin albedo. A fluorinated ethylene propylene (FEP) cap was placed over the excitation fiber to use as a reference material in the normalization of spectra for comparison.

The in-vivo transcutaneous Raman spectra were collected in standard formats. Spectra were pre-processed for removal of cosmic spikes and correction of spectrograph/detector alignment and grating-induced anamorphic magnification (curvature). Spectra were corrected for the fluorescence background by fitting background to a low order polynomial (Polynomial order = 5). Overlapped bands are fitted to mixed Gaussian–Lorentzian functions. Band heights and areas are measured.

After preprocessing, information was extracted about the various components of bone using bands and band intensity ratios listed in Table 1. Commonly, in bone, Raman spectroscopy positions of mineral and matrix bands and ratios of band heights or band areas are reported [19]. The height of the intense $PO_4^{-3}$ $\nu_1$ stretch (958 $cm^{-1}$) was used as a measure of mineral content and the width of this band was used as a measure of mineral crystallinity. The height of the amide I band (1660 $cm^{-1}$) or phenylalanine band (1001 $cm^{-1}$) was used as the measure of matrix content. Mineral to matrix ratio (MTMR) is the intensity of the bone mineral band divided by the intensity of a matrix band. Standard principal component analysis based multivariate methods were used to unmix bone and overlying tissue spectra returned by our measurements. It is important to note that the Raman instrumentation